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BIPOLAR HEAD REGENERATION IN PLANARIA INDUCED BY CHICK EMBRYO EXTRACTS[1]

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When a planarian is transected the anterior cut end will regenerate a head while the posterior end will give rise to a tail. Child (1941), postulated in his axial gradient theory that quantitive axial gradients of physiological activity somehow account for axial patterns of differentiation. This theory predicts that the higher level of physiological activity at the anterior cut end leads to the formation of the head at that site. An alternative suggestion is that polarity is maintained by the presence of specific inhibitors which similarly follow an axial gradient pattern. When a planarian is decapitated, the brain forms before the eye spots or auricles. However, regeneration of the brain does not occur if another brain is present (Morgan, 1902; Rand and Ellis, 1926; and Miller, 1938). Such effects could be due to specific inhibitors or they could result from a metabolic competition. Decapitated planarians reared in water containing centrifugal supernatants of head homogenates had smaller brains, or the brains were absent, suggesting there are specific inhibitor substances (Lender, 1960).

The aim of this investigation was to determine if polarity is determined by the presence of specific inhibitors or if it is the function of an axial gradient of physiological activity. Regeneration was allowed to occur in the presence of centrifugal supernatants of either heads or tails of planaria, *Dugesia dorotocephala*, to determine if these fractions possess inhibitory activity. In order to test the idea that competitive metabolic processes may play a role in determining polarity, small cut pieces of planaria were cultured in chick embryo extract and ultrafiltrate to see if these nutrients provided in these preparations could alter the normal regeneration polarity by inducing the formation of bipolar heads.

Materials and Methods

Planarians, *Dugesia dorotocephala*, which were 1–2 centimeters in length were maintained in aerated tap water in covered containers containing 100 units of penicillin and streptomycin per milliliter and were starved for at least 5–7 days prior to use in an experiment. Before cutting, the worms were washed several times with boiled tap water, then while beneath a shallow layer of water they were exposed to bacteriocidal ultraviolet light for five minutes and then cut with sterile single edge razor blades. In obtaining the centrifugal supernatants for the inhibitor experiments the worms were cut into three equal parts, thus providing the head, mid-body and tail fractions. The middle piece always included the

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pharynx. The protein concentrations of the centrifugal supernatants were determined by the Lowry method (Lowry, Rosebrough, Farr and Randall, 1951). The chick embryo extracts used in these experiments were obtained in the frozen state from Grand Island Biological Company. The effect of the chick embryo fractions upon DNA synthesis in pieces of planarians was tested by exposing pooled segments of planaria to 25 μ c/ml of H^3-thymidine in 2 ml of the chick embryo extract solutions and in a 1/10 dilution of a balanced salt medium (Niu-Twitty, 1953). At the conclusion of the incubation period the worms were washed five times with boiled tap water. The worms were then homogenized in cold 7% trichloroacetic acid (TCA) and the pellet washed three times by centrifugation. The residues were extracted twice with 1:1 ethanol-ether, twice with 1:4 ethanol-ether and once with ether alone to remove lipids; the residues were then dried. The diphenylamine method (Dische, 1955) was used to determine DNA concentration and levels of labeled DNA were estimated with a liquid scintillation counter.

Results

Experiments with centrifugal supernatants

The head, tail and midbody centrifugal supernates used for the inhibitor experiments were prepared by homogenizing at 4° C in aerated tap water containing 50 units of penicillin and sterptomycin per milliliter. Homogenates were centrifuged at 10,000 × g for 30 minutes at 4° C and the supernatants were collected. The supernatants were passed through sterile millipore filters of 0.45 and 0.22 μ pore diameter before use. Heads were removed from a group of 25 worms by cutting just posterior to the auricles and the decapitated worms were exposed for 9 days at 20° C to supernatants of homogenized head, tail and midbody sections at concentrations between 2 and 100 pieces per two milliliters of homogenation medium. In terms of protein concentration, cut planaria were exposed to centrifugal supernatants from head regions with a protein concentration of 6.05 μg/ml to 605 μg/ml and to centrifugal supernatants from tail regions with protein concentrations of 4.58 μg/ml to 458 μg/ml. Fresh supernatants were prepared daily and the media changed each day of the nine day exposure period. Control and test worms were observed daily for the appearance of eyespots and auricles. After nine days of culture, test and control worms were fixed at which time both eye spots and auricles had appeared. They were then embedded in paraffin, 5 μ serial sections made and sections were stained with 0.1% cresyl violet.

All planaria exposed to head and tail concentrations of 2 through 87 pieces per two milliliters survived the nine days of incubation and regenerated normal eyespots and auricles. Tail and head concentrations of 100 pieces/2 ml killed test worms within 48 hours after first exposure. In control and test worms incubated in non-lethal supernatant concentrations, auricles appeared between 48–72 hours, detectable eyespots between 60–96 hours, fully discernable eyespots between 85–120 hours and fully regenerated heads by 6 days. There was no significant delay in time of appearance of the eyespots in the centrifugal supernatant fractions. Serial sections of control and test worms showed the presence of brains of equal length (175 ± 5 μ) in both the control and test worms (Fig. 1).

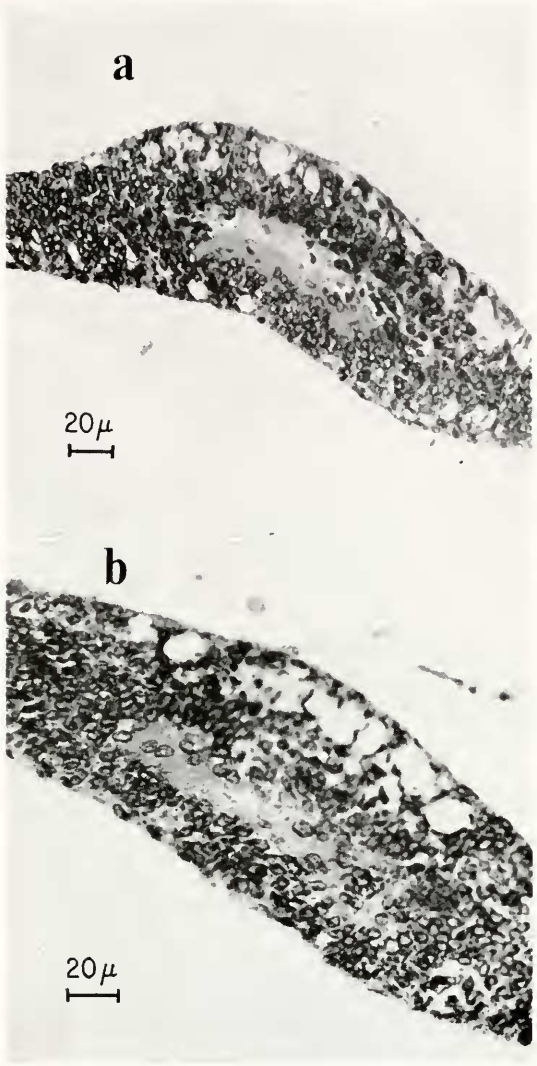

FIGURE 1. Transverse sections of heads that formed after 9 days of regeneration in (a) bubbled tap water and (b) a centrifugal supernate obtained by homogenizing 50 planarian heads in 2 ml of bubbled tap water. The more lightly stained tissues in the center of the sections are the brains.

Effect of chick embryo extract on polarity

In a preliminary experiment, to determine what body levels and which nutrient medium produced bipolars, the planaria were transversely cut at 8 body levels (Fig. 2) and the cut segments of worms allowed to regenerate in chick embryo extract and chick embryo extract ultrafiltrate. Regenerating pieces from posterior levels 5 and 6 cultured in 1% chick embryo extract and 1% chick embryo extract

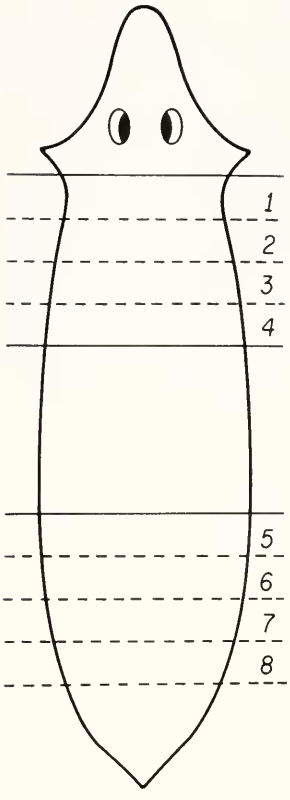

FIGURE 2. The levels at which worms were cut for the experiments with chick embryo extract.

ultrafiltrate in 1/10 Niu-Twitty saline (Niu and Twitty, 1953) were the only levels showing bipolar head regeneration, *i.e.*, heads forming at each end of the cut segments of the worms. Segments of worms from regions 1 to 4 and 7 to 8 regenerated heads at the anterior end and tails at the posterior ends. No bipolar heads arose in pieces cultured in saline alone and these pieces formed heads at the anterior end surface and tails at the posterior end.

In further experiments, twenty sections each of posterior body levels 5 and 6 from worms starved for 5, 10, 20 and 30 days were cultured in saline and nutrient media. The sections were exposed to test solutions for 4 days, after which regeneration was completed in 1/10 saline (Table I). No bipolar head regeneration occurred in the short posterior pieces from worms starved 5–10 days.

However, 6 bipolar heads out of 26 cut pieces regenerated from cut posterior level 5 pieces and 8 bipolar heads out of 21 cut pieces were formed in posterior level 6 cut pieces in nutrient media from worms starved for 20 days. After 30 days of starvation, 9 of 34 level 5 pieces formed bipolar heads in nutrient media, while 7 of 25 level 6 pieces in nutrient media regenerated bipolar heads. None of the posterior level pieces produced bipolar heads when the medium was 1/10 Niu Twitty saline (Table I). A typical regenerate with a bipolar head is shown in Figure 3.

TABLE I

Effect of nutrient media on incidence of bipolar head formation

Medium	Level of worm	Number of bipolars / Number of surviving sections = %			
		Days of starvation			
		5	10	20	30
(1.) 1/10 Niu-Twitty saline +1% chick embryo extract	5	0/20 = 0	0/19 = 0	4/10 = 40%	4/16 = 25%
(2.) 1/10 Niu-Twitty saline +1% chick embryo extract ultrafiltrate	5	0/19 = 0	0/19 = 0	2/16 = 12.5%	5/18 = 27.8%
(3.) 1/10 Niu-Twitty saline	5	0/20 = 0	0/18 = 0	0/16 = 0	0/14 = 0
(1.) 1/10 Niu-Twitty saline +1% chick embryo extract	6	0/20 = 0	0/19 = 0	3/5 = 60%	3/12 = 25%
(2.) 1/10 Niu-Twitty saline +1% chick embryo extract ultrafiltrate	6	0/20 = 0	0/19 = 0	5/16 = 31.25%	4/13 = 30.7%
(3.) 1/10 Niu-Twitty saline	6	0/20 = 0	0/17 = 0	0/19 = 0	0/15 = 0

Isotopic experiments with worms exposed to chick embryo extract

It is possible that the chick embryo extract causes bipolar head regeneration in short posterior pieces from starved planaria by stimulating cell division. In order to determine if the chick embryo extracts had stimulated DNA synthesis and cell division in the regenerating sections an experiment using H^3-thymidine was performed. One hundred and fifty worms starved for 30 days were cut into

FIGURE 3. A posterior section of a worm which regenerated a head at each end after culture in 1% chick embryo extract.

head (levels 1–4), midbody (between levels 4 and 5) and tail pieces (levels 5–8) (Fig. 2). Fifty such sections of each level were each incubated in two nutrient media and saline. To each of the media H^3-thymidine was added to a final concentration of 25 μ c/ml and cut worms were incubated for 24 hours. The results of this experiment clearly demonstrate a stimulation of DNA synthesis of all three levels of the pooled cut worms which had been cultured in nutrient medium (Table II). There was a greater stimulation of DNA synthesis at middle and posterior levels than at anterior levels. Those cut pieces giving rise to bipolar heads were found using posterior levels of cut worms (Table I).

TABLE II

Effect of nutrient media on DNA syntheses in planaria starved for 30 days. Fifty cut pieces were incubated in 25 μ c/ml H^3-thymidine for 46 hours

CPM / MG DNA

Medium	Level of worm			% stimulation DNA synthesis compared to controls		
	Anterior	Middle	Posterior	Anterior	Middle	Posterior
(1.) 1/10 Niu-Twitty saline + 1% chick embryo extract	21,400	16,756	14,872	19.4	37.9	32.1
(2.) 1/10 Niu-Twitty saline + 1% chick embryo extract ultrafiltrate	18,460	15,539	12,600	13.7	33.1	19.8
(3.) 1/10 Niu-Twitty saline-control	17,238	10,391	10,100	—	—	—

DISCUSSION

According to Lender (1960) decapitated planarians reared in water containing centrifugal supernatants of head homogenates either did not regenerate brains, or the brains were of reduced size. Lender felt that the diffusion of inhibitory substances from the brain gave rise to an anterior-posterior gradient of inhibition. In recent work on inhibitors Ziller-Sengel (1967a, 1967b) has reported that the pharyngeal region of the planarian *Dugesia lugubris* contains a specific inhibitor which delays the regeneration of the pharynx. The inhibitor is present in filtered extracts of the pharyngeal region and is species-specific since homogenates of the pharyngeal regions of *Dugesia tigrina* do not inhibit regeneration of the pharynx in *Dugesia lugubris*.

The results of this investigation do not demonstrate the presence of a specific inhibitor of brain regeneration in 10,000 $\times$ *g* supernatants of heads of *Dugesia dorotocephala*. Centrifugal supernatants of homogenates from the three body levels at concentrations of 2 to 87 cut sections in 2 milliliters of homogenization medium (6–600 μg protein/ml) do not exhibit inhibitory activity.

Brondsted (1955) showed that a head regenerates in a window cut out of the fore part of the planarian, *Bdellocephala*, when the old head is present. This implies that inhibitory substances from a certain body structure do not travel through the body to inhibit the regeneration of identical structures. Brondsted's

experiment, and the reports of Ziller-Sengel (1967a, 1967b), showing a delay in the regeneration of the pharynx and not a loss or a reduction in size of the regenerated organ, do not support the role of specific inhibitors in maintaining polarity.

Exposure of posterior cut sections of planaria to nutrient media (chick embryo extract and chick embryo extract ultrafiltrate) after a sufficient time of starvation of the worms does alter the polarity of the regenerating pieces. It has been shown that cephalocaudal physiological gradients exist in planaria and that polarity of regenerates can be reversed by abolishing such gradients (Flickinger, 1959; Flickinger and Coward, 1962; Coward, 1968). Nutrient media (Coward, Flickinger and Garen, 1964) accelerate both the rate and extent of regeneration in posterior regions of cut starved planarians. Although very short cut pieces often form heads at both ends (Janus heads), this did not occur in the control pieces in saline. The levels of the worm just posterior to the pharynx (levels 5 and 6) are in the fission zone of the planarian and thus may account for bipolar head formation in this region.

It has been demonstrated that inhibition of DNA synthesis is necessary in order to produce bipolar head regeneration with cloramphenicol (Kohl and Flickinger, 1966). Therefore, it seemed important to learn if a stimulation of DNA synthesis, and presumably cell division, occurred when bipolar head formation is induced by stimulation, rather than by inhibition. The results of the H^3-thymidine incorporation experiments in which anterior, middle and posterior segments were regenerating in saline show that after 30 days of starvation there still exists a cephalocaudal gradient of DNA synthesis as described by Coward and Flickinger (1965), and Kohl and Flickinger (1966). However, no difference in DNA synthesis existed between the pooled middle and posterior regions. Furthermore, posterior body levels showed a greater increase in the percentage of stimulation of DNA synthesis with nutrient media than does the anterior level, indicating a greater stimulation of cell division in these levels than in the head level.

The results of this investigation, and the reported work of previous investigators, strongly suggest the presence of a cephalocaudal metabolic gradient in planarians in which metabolic competition results in the dominance of anterior levels over each succeeding posterior level. No evidence for specific inhibitor substances was found in this study.

Summary

1. Decapitated planaria, *Dugesia dorotocephala,* exposed to head and tail centrifugal supernatants for nine days do not exhibit inhibition of brain regeneration. The experimental and control worms had brains of equal sizes.

2. A significant number of bipolar heads regenerated in small pieces from the posterior region of starved worms which were cultured in chick embryo extract and chick embryo extract ultrafiltrate.

3. Chick embryo extracts produce a stimulation of DNA synthesis in pieces of starved worms which were cut into anterior, middle and posterior sections. There is a greater stimulation at the posterior levels which can give rise to bipolar heads. This stimulation of DNA synthesis is thought to reflect an increase in cell division at these levels caused by the chick embryo extract.

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